



DETECTION OF THE DIVERSITY OF *PSEUDOMONAS AERUGINOSA* PHAGES ISOLATED FROM DIFFERENT SOURCES

Noura B. Abdulrahman^{1*}, Akeel H. Al-assi¹ and Ali S. Al-Jibory²

¹Biology Department, College of Science, University of Tikrit, Iraq.

²Biology Department, College of Scienc, Al-Iraqia University, Iraq.

Abstract

Pseudomonas aeruginosa phages are widespread in the environment. In this study numerous types of phages were collected from different sources in order to show their distribution and diversity in Tikrit. The mean for detection diversity was phenotypic tests such as phages' tolerance for: host infection, acidity and alkalinity, chloroform, sunlight and temperature, while the genotypic detection included using 18 primers for Randomly Amplified Polymorphic DNA (RAPD-PCR). The aim of using RAPD-PCR is to detect unidentified phages' diversity and also to obtain randomly single band to exploit it for sequencing and knowing its content. The results showed obtaining eight lysogenic different phages with a high diversity and the ability to isolate their genomic DNA manually, then sequence one RAPD-PCR product for two different phages.

Key words: *Pseudomonas aeruginosa*, phages, diversity, RAPD-PCR, phenotypic, genotypic, sequencing

Introduction

Bacteriophages are bacterial viruses, widespread in the environment and are recognized as the most abundant biological agent on earth. They are very diverse in morphology, size and genomic organization (Kasman and Porter, 2019). Phages are the most numerous of all viruses in the environment and are estimated to be globally more numerous than bacteria. This abundance plays important parts in the evolution of bacterial communities and might influence global biogeochemical cycles. A limited number of studies have investigated phage distribution in natural environments (Batinovic *et al.*, 2019). *P. aeruginosa* is a gram-negative bacterium, it is an opportunistic pathogen of humans; abundantly, is widespread in the nature and commonly present in moist environments of hospitals. It causes disease in humans with abnormal host defenses (Török *et al.*, 2017). In hospital, the bacterium is the leading causes of nosocomial lung infections and a common causer of wound infections, especially of thermal burns (Riedel *et al.*, 2019; Ceysens and Lavigne, 2010; Ryan and Ray, 2014). To date, about 6000 different phages had been visualized by electron microscopy, among which around 10% specifically target members of the *Pseudomonas* genus. Historically, *Pseudomonas* phages have been used for the epidemiological locating of specific

strains; *e.g.*, the *Pseudomonas aeruginosa* typing phages of the Lindberg set and as tools in molecular biology (Ceysens and Lavigne, 2010). It was improved by the continuous advances in whole-genome sequencing, the number of sequenced *Pseudomonas* phage genomes available in public databases increased (essentially small ssDNA phages) complemented by several complete and partial (cryptic) prophage sequences, contained within the host chromosome (Roy *et al.*, 2010; Mavrodi *et al.*, 2009; Battle *et al.*, 2009). Depending on previous studies, it was found that the phages which infect *Pseudomonas aeruginosa* belong to different families: Myoviridae, Podoviridae (Latz *et al.*, 2017) and Siphoviridae (Essoh *et al.*, 2015); which their genomic diverse between ssDNA, dsDNA, ssRNA and dsRNA.

Material and Methods

Bacterial Strains

The bacterial strains included 60 previously identified *Pseudomonas aeruginosa* from different clinical sources in Tikrit city.

Bacteriophage Isolation and purification

Eight samples of phages were collected from different sources by 500 ml containers: sewage sludge, which were obtained from the waste water treatment facility located in Sheishein area at Tikrit- Salah al din Governorate, Tigris

*Author for correspondence : E-mail : noura_bda@st.tu.edu.iq

river, Salah-Addin hospital soil and from cow, sheep and fowl feces in different places of Tikrit city (Mohammed-Ali and Jamalludeen, 2015; Zablocki *et al.*, 2016). The liquid samples were aseptically filtered by 0.8 µm pore sized cellulose filter to eliminate particles debris followed centrifugation at 2500rpm for 10min. The supernatant was finally filtered by 0.45 µm pore sized filter to remove bacterial cells and cellular debris. Whereas the solid samples were solved in sterile distilled water in 25 ml tubes, shaking in water bath for half hr., then centrifuged at 2000 rpm for ten min and the same steps that mentioned above were done.

In order to detect *P. aeruginosa* phages, five ml of the bacterial strains in Luria Bertoni broth (LB), which prepared according to (Atlas, 2006) by solute Trypton 10g (HIMEDIA, India), Yeast extract 5g (HIMEDIA, India) and NaCl 10g in 1000 ml of D.W) were mixed with 45 ml of previously prepared phage suspension and 5ml of sterilized LB broth. Then, it was incubated at 37°C with shaking at 80 rpm for 24 hrs. At the end of incubation period, the suspension was clarified by centrifugation at 2500 rpm for 10 minutes and the supernatant was filtered through 0.22µm pore sized filters to decline bacteria. The suspension of expected phages was kept at 4°C as a stock solution for the next step (Ferman and Jameel, 2019).

Briefly, phage purification was done by diluting its suspensions; a 100 µl of diluted phage and 100 µl host bacterium (10⁹CFU/ml) were mixed with 5 ml of LB soft agar (0.75 % agar, w/v) and poured quickly on the previously solidified LB agar plate. Then, plaque numbers were counted after overnight incubation at 37°C (Alsaffar and Jarallah, 2016).

After Making double layer agar test, the midpoint of the plaques was scraped off by sterile tip or inoculation loop and moved to sterile LB broth that contains the appropriate strain and incubated overnight at 37°C. Later, the mixture was centrifuged at 5000 rpm for 25min and filtered through Millipore Membrane Filter (0.22µ). The phages then saved in sterile dark bottles in the refrigerator for propagation in the same mentioned method to certify their purity and for next experiments (Ferman and Jameel, 2019).

Phages physical and chemical properties

Acidity and Alkalinity

Phages tolerance to acidity and alkalinity experiments were done according to (Jamalludeen *et al.*, 2007). A 100 µl volume of phage suspension (10⁹PFU/ml) was added to 900 µl of saline adjusted to series of pH (3-10). The mixture was incubated

at 37°C for 48 h. and checked every 4 h. The titer of the surviving phages was detected by plaques by doubled layer method.

Chloroform

This experiment was done according to (Jameel, 2016) by exposing the phages to serial concentration of chloroform (10, 30, 50, 70 and 100) v/v.

Sunlight

A ten milliliters of phage suspension (10¹⁰PFU/ml) was exposed to direct sunlight at room temperature for seven days' light continuously. A 100 µl of the sunlight exposed phage was tested after every day to detect PFU (Chandra *et al.*, 2011).

Temperature

Phage suspension tolerance was tested in multi temperature (4, 37 and 100) and the aliquot of phage was taken every 10 min for 1hr, and tittered by double-layer method (Hamza *et al.*, 2016).

Phage's DNA Extraction

Phages' DNA were extracted by Phenol: Chloroform: Isoamyl in the ratio of 25:24:1. In this protocol the extraction of genomic DNA of phages was in a lysate, that contains residual of media components, cell wall debris, flagella, nucleic acids, bacterial proteins and unassembled phage proteins in addition to the phage themselves. The steps of phages extraction were done after precipitation with Poly-ethylene glycol PEG (10%) according to the Center for Phage Technology (CPT, 2020).

RAPD-PCR Primers and Program

The primers that were chosen in this study are illustrated in table 1. The program of RAPD-PCR was communal for the all used primers: 94°C (4min) for initial denaturation, 93°C (45sec) denaturation, 38°C (45sec) annealing, 72°C (90sec) extension and 72°C (7 min) for final extension.

Table 1: RAPD-PCR primers.

No.	Primer	Sequence 5'→→→3'	No.	Primer	Sequence 5'→→→3'
1.	OPA-01	CAGGCCCTTC	10.	OPC-16	CACACTCCAG
2.	OPA-06	GGTCCCTGAC	11.	OPC-10	TGCTCTGGGTG
3.	OPB-04	GGACTGGAGT	12.	OPD-03	GTCGCCGTCA
4.	OPB-12	CCTTGACGCA	13.	OPD10	GGTCTACACC
5.	OPB-14	TCCGCTCTGG	14.	OPD-18	GAGAGCAAC
6.	OPC-08	TGGACCGGTG	15.	OPG-02	GGCACTGAGG
7.	OPH-16	TCTCAGCTGG	16.	OPG-08	TCACGTCCAC
8.	OPJ-04	CGGAACACGG	17.	OPG-14	GGATGAGACC
9.	OPB-20	GGACCCTTAC	18.	OPY-04	GGCTGCAATG

Results and Discussion

After incubation the expected phage samples with *Pseudomonas aeruginosa*, isolation them by filtering and testing them to find the presence of phages by doubled layer agar method Fig. 1, it was found that the eight distinguished phages were with lysogenic life cycle, which gives an impression that the most prevalent *P. aeruginosa* bacteriophages in Tikrit city are lysogenic.

To know the host range of the isolated bacteriophages, spot test was applied for all bacterial strains Fig. 2 and the infection percentage for each phage is demonstrated in table 2, which supposes that the most prevalence phages are Ph5 then Ph9 and Ph11 respectively. It should be mentioned that only one bacterial strain did not be infected with any of these eight phage.

One-step growth curve

Approximately, the results showed that all phages have latent period 20 min, then increases linearly with time till about 45 minutes. After 45 minutes it stays nearly constant. Phage Group A: (Ph1 and Ph5) release takes place uniformly during 25 minutes; so, the burst size of these Phages is 130 (the result found through dividing the concentration of bacteriophage at t = 45 by the concentration of bacteriophage at t = 25. While Group B:

Table 2:

Phages	Phage's source	Zone diameter mm.	Isolates percentage that were infected with phage
Ph1	Shishin Sewage	1.5	18.3%
Ph2	Tigris river	2.5	33.3%
Ph4	Hospital soil	2	13.3%
Ph5	Cow feces	1.5	76.7%
Ph9	Sheep feces	1	73%
Ph10	Fowl feces	8	25%
Ph11	Road sewage	2	60%
Ph15	Infected isolate	3	35%

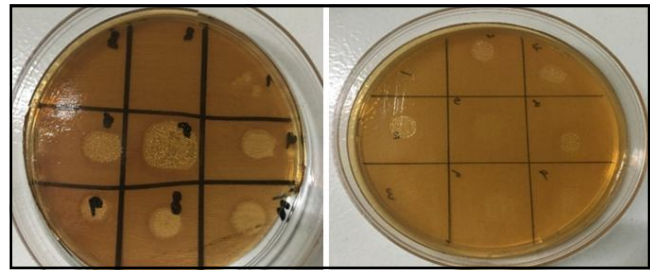


Fig. 2: Bacterial typing by bacteriophages.

(Ph2, Ph9 and Ph10, Ph11) release takes place uniformly during 35 minutes; so, the burst size of these phages is 94. The phages Group C: (Ph4 and Ph15) have latent period near to 40 min, with burst size 70 at t = 45 as they are clarified in chart 1.

Stability to pH

After exposing the phages to gradient degrees of pH (3 to 10) for two days; All phages were resistant to pH 5 to pH 9 except Ph15, which was sensitive to pH 9. Titration were done chart 2, no phage was detected after the treatment with pH 3, 4, 9 and 10 after 24hr. However, all remained infectious after treatment with pH 5, 6, 7 and 8 till 48 hr. Viruses are usually stable between pH values of 5.0 and 9.0. Some (for ex: entero-phages and environmental phages) are resistant to acidic conditions, but all are destroyed by alkaline conditions (Brooks *et*

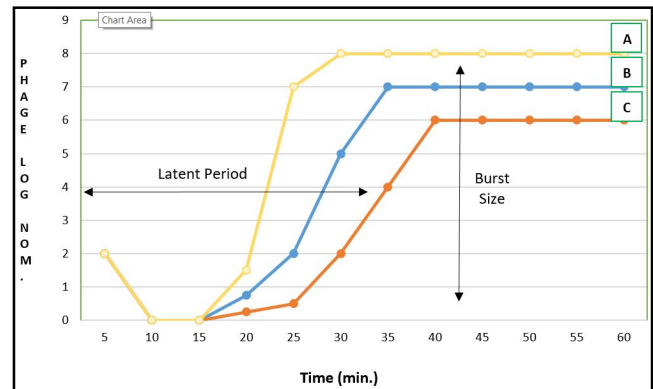


Chart 1: One-step growth curve.



Fig. 1: Different pattern of the isolated bacteriophages from diverse sources.

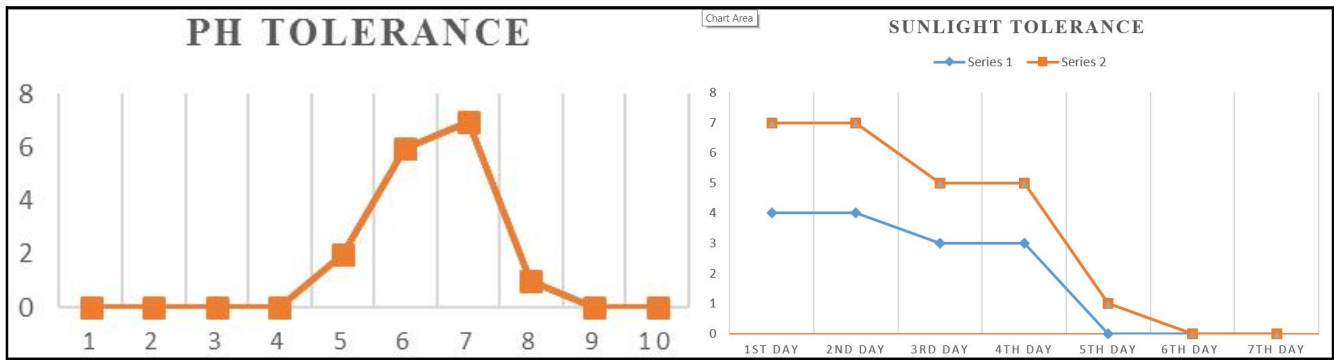


Chart 2, 3: Phages' stability to the pH and sunlight.

al., 2013). The results were close to (Alsaffar and Jarallah, 2016), who showed that their isolated phage particles were stable and survived at pH values between 6 and 8. The pH stability of phages is important for microbiological interest, for example, their survival in the variable pH environment of gut and also for practical purposes related to the possible therapeutic uses.

Sunlight

The number of phages decreased during the first 24 hr. of exposure to sunlight and afterwards, the count was diminished to 0 by the 5th day for most isolated phages, while some of them diminished to 0 after the sixth day chart 3. Jagdale *et al.*, 2019) found that the isolated bacteriophages when were exposed to direct UV radiation for 30 min showed a reduction in the titer to 89.36% by PRxL2 and 96% by SSxL8. The data suggests that phages were able to survive the UV radiation because originally, they were isolated from sunlight exposed environment.

Chloroform

By exposing the phages to serial concentration of chloroform (10, 30, 50, 70 and 100) v/v, results showed that the ability of phages to grow in the all mentioned concentration, which agreed with (Jameel, 2016).

Stability to Temperature

Thermal inactivation point of phages is important

parameter to be determined for their identification and classification. For example, phages with a high degree of thermos-stability have better chance of survival in organic composts, in which temperature may exceed 70°C. the other benefit is to be used as a biological disinfectant to control *P. aeruginosa* in the environment (Yang *et al.*, 2016). Whatever, results showed that most phages have infectivity and the ability to grow at temperature between (-20° to 40°) after 24 hr. and some of them 48hr. The results agreed partially with (Alsaffar and Jarallah, 2016). who explained that phages able to grow in gradient between (30°-45°). The variability in heat tolerance in the viruses generally due to their structures; Icosahedral viruses tend to be stable, losing little infectivity after several hours at 37°C. Enveloped viruses are much more heat-labile, rapidly dropping in titer at 37°C. (Jagdale *et al.*, 2019) reported that their phages were stable over a broad temperature range from 4-50°C as compared to the phages reported earlier.

RAPD-PCR Results

Randomly amplified polymorphic DNA (RAPD)-PCR were applied by using 18 primers in order to measure the genetic diversity of phages and evaluate whether they are related with each other or not. All primers gave reaction results except one primer which was OP D-03 Fig. 3.

Among other DNA-based approaches, random PCR



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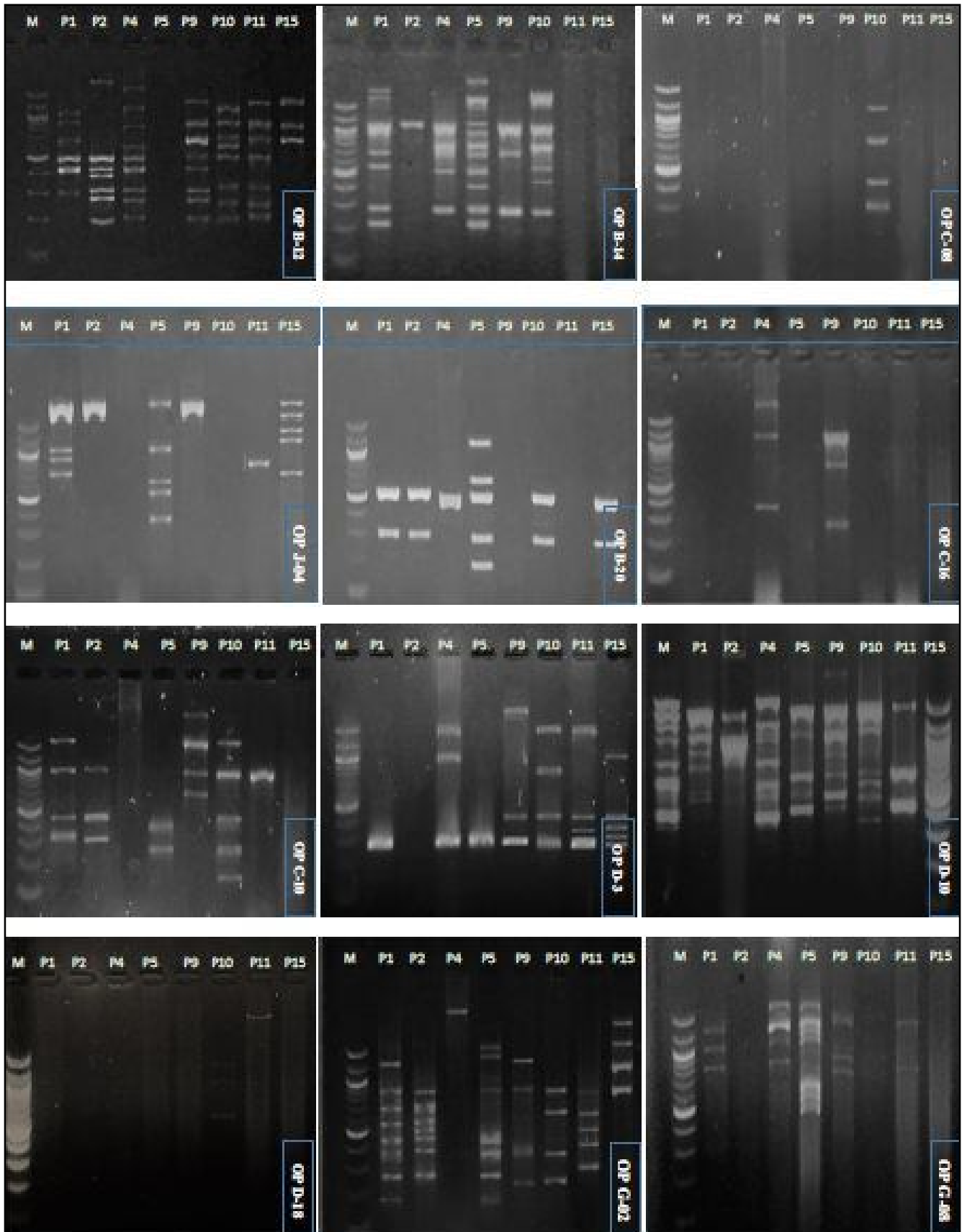


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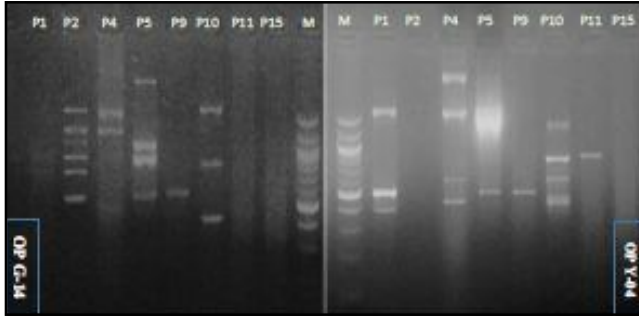


Fig. 3: RAPD-PCR Results for 8 Bacteriophages.

amplifications of DNA segments using short primers of random nucleotide sequence have been used to generate specific profiles or genomic fingerprints that are used to compare the genotypic diversity among organisms, for example, bacterial isolates or whole bacterial communities and even phages, like *P. aeruginosa* phages (Gutiérrez *et al.*, 2011).

RAPD reactions are so sensitive and changeable if any component concentration or any condition of the reaction were altered; then, result achievement is non-reproducible without optimization (AL-Asie, 2002). In RAPD analysis, Band density is a regarded characteristic if the optimization was achieved, it explains the variance of copy number in organisms. Moreover, it could be relied on number of the produced bands, their different molecular weight, shining intensity and presence or absence of them in different studied samples (Lanzone *et al.*, 2016; Altaie, 201). All these varieties are providing evidence of high genetic diversity and polymorphism among experimental samples (Gaber *et al.*, 2018).

Bands presence or absence due to possession that primer of the complementary sites which enable it to bind with the studied genome or not; according to that, varieties between samples appear and so, the importance of band presence is equivalent to its absence. The second rule in primer binding depends on the size of the studied genome and primer sequence, which differ in binding by relying on alteration even in one nucleotide in same primer or genome (AL-Asie, 2002).

The differences in the genome are normally found between already diverse species or in the related individuals, due to recombination during cell division or by mutations: (insertion, deletion and substitution) in genome where the sites of primer binding are existent; that what causes alteration in the arrangement of the remain nucleotides, then prevents primer attachment at its specialized site on genome (Williams *et al.*, 1990). In this manner, the absence of main band in one sample without the other is regarded an important marker to

distinguish the genetic distance of this sample. Besides, the dissimilarity in molecular weight (band size) and the number of bands is also another rule that RAPD depends on to find genetic diversity. The variance in band size is due to the distance between two binding sites of the primer, that will be polymerized in every cycle of the PCR (AL-Asie, 2002).

The results of all RAPD primers were achieved in evaluating the genetic distance for the studied phages by special application on computer; they showed that the minimum genetic distance was 0.443, which appeared between P9 and P11; whereas the maximum genetic distance was 0.971, that appeared between P4 and P15, as its shown in table 3. According to the genetic distance data, a dendrogram for all phages was prepared; two main clusters appeared (A and B), which included other sub-clusters as its presented in chart (4-4). The first sub-cluster (A1) included the phages: p1 and P5 with 0.562 of genetic distance. The second sub-cluster (A2) included two groups: A21 and A22; each one was also divided into subgroups, which totally comprised of: Ph2, Ph4, Ph9, Ph10 and Ph11. The second main cluster (B) included P15 only.

After comparing the RAPD results as a bacteriophages genotyping and their phenotyping, it had been noticed a clear correlation between them. For example: phenotypically, in one step growth it was

Table 3: Genetic distance values for the studied bacteriophages according to RAPD results.

	1	2	4	5	9	10	11	15
1	0.000							
2	0.580	0.000						
4	0.833	0.708	0.000					
5	0.562	0.729	0.868	0.000				
9	0.686	0.693	0.625	0.782	0.000			
10	0.692	0.583	0.624	0.743	0.544	0.000		
11	0.747	0.625	0.695	0.862	0.443	0.505	0.000	
15	0.848	0.900	0.971	0.955	0.674	0.752	0.650	0.000

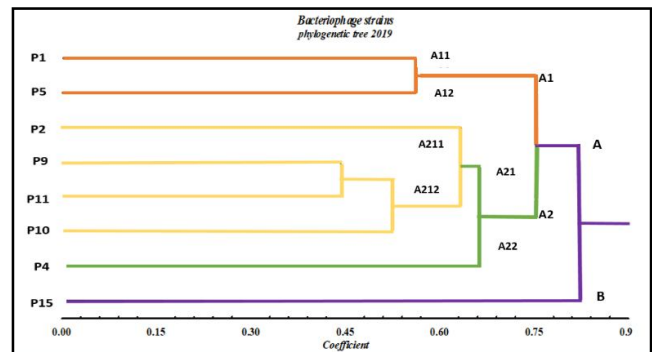


Chart 4: phylogenetic relationships according to RAPD results.

appeared three groups of bacteriophages for latent period Group A: (Ph1 and Ph5), Group B: (Ph2, Ph9 and Ph10, Ph11) and Group C: (Ph4 and Ph15). These groups were close to the divisions of the main cluster in the phylogenetic tree except P4. As well as; in pH tolerance estimation for bacteriophages, Ph15 on the contrary of the other phages was distinctive in its inability to tolerate pH 8; that what agreed its genetic distance from the other phages. Temperature tolerance also showed the same for Ph15, which was unable to live at -20 for 48hr., while the other phages did. The phages Ph9 and Ph11 showed a high similarity in living at 50°C and closeness to Ph2, what enhances the RAPD results; on the other hand, Ph9 and Ph11 showed also convergent percent in bacterial strains infection.

Sequencing for phages

Two samples of bacteriophages (Ph1 and Ph5) were sequenced after getting single band close to 300 bp by the RAPD-PCR primer (OP C-10), in order to get more information about these phages. The PCR product of them was randomly anonymous gene, so the sequencing results gave us the gene type: *uvrD*, that codes for helicase enzyme, which is responsible of unwinding double helical DNA [31]. These two sequenced fragments of

the phages were identical to each other 100% and share the same gene of *Proteus mirabilis* in 99.55%, which might ensure transformation between bacteria on hand and transduction between bacteria and their bacteriophages on the other hand. Due to the other phages have this band in same site, it's so possible that all of those phages have this same necessary gene (*uvrD*). No variation between these sequenced segments Fig. 4, 5; each nucleotide amount in each two phages strain for *uvrD* are clear in table 4.

From the mentioned phenotypic behaviors and genotypic results of the studied phages, it should be noticed a clear diversity, which might due to their different environments, host strains and might due to evolution that resulted by reaction between the genetic material and environment.

Conclusions

According to the results, the most spread phages in Tikrit city are lysogenic types with an observed diversity, which are supposed to enable bacteria in evolution by transduction. The RAPD-PCR gives variety for the studied bacteriophages by: unique, absent and polymorphic bands that were not enough to be detected by phages' physical and chemical tests.

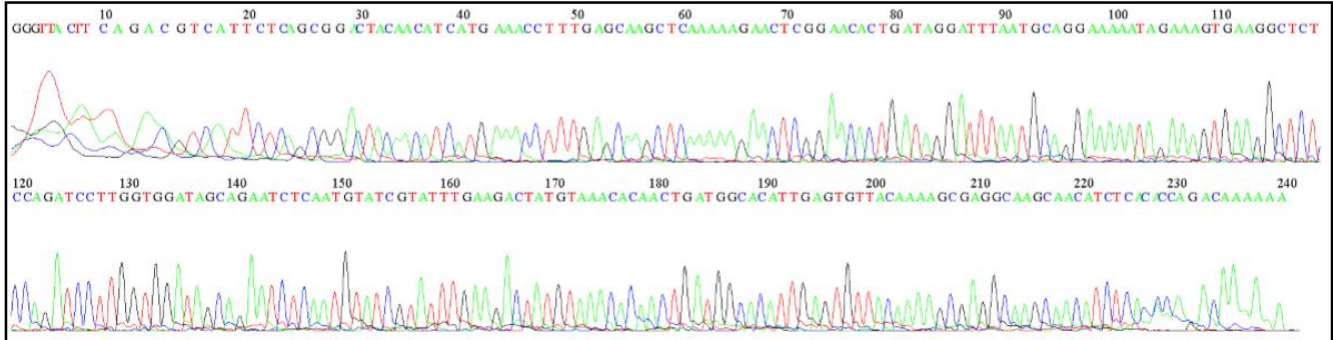


Fig 4: Sequencing results for Ph1.

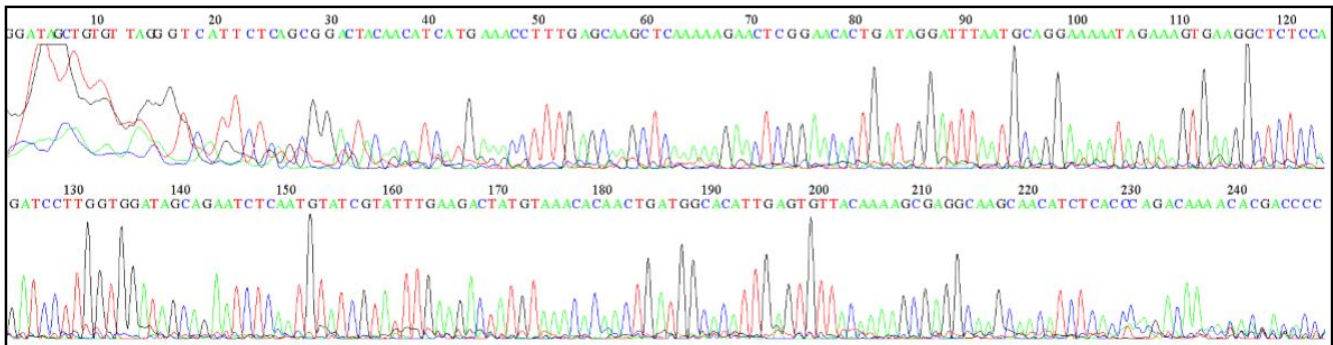


Fig 5: Sequencing results for Ph5

Table 4: Nucleotides amount and type according to their arrangement in the codon for *uvrD*.

P	T1	T2	T3	TA _v	C1	C2	C3	CA _v	A1	A2	A3	AA _v	G1	G2	G3	GA _v
Ava.	17	21	29	22.3	19.7	25.8	10.8	18.8	28.8	36.4	49.2	38.1	34.8	16.7	10.8	20.8

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